SUMMARY

The nitrogen cycle in nature is essentially driven by prokaryotic microorganisms. Nitrogen is one of the most important elements for the synthesis of cell material; it accounts for approximately 14% of dry weight. All eukaryotes and the majority of the prokaryotic organisms are dependent on a source of combined nitrogen. Only a limited number of bacteria are able to grow with molecular nitrogen. The crucial reactions in the biological cycle of nitrogen are denitrification and nitrogen fixation. Denitrification is the process which finally results in the conversion of bound nitrogen into molecular nitrogen. Thus, denitrification presents a continuous loss of bound nitrogen. Biological nitrogen fixation by prokaryotes is the only process that counteracts these losses.

In many environments the availability of a source of combined nitrogen limits the growth of organisms and especially primary production. Seawater and sandy sediments of intertidal zones that consist of almost pure quarz are very poor in combined nitrogen. The aim of this thesis was to study the role of nitrogen fixation in the development of populations of cyanobacteria in an intertidal zone and the physiological adaptations of cyanobacteria which enable them to maintain nitrogenase activity under aerobic conditions.

The intertidal flat that was investigated is situated on the island of Mellum, southern North Sea. On the west beach of the island large areas were covered by benthic cyanobacteria, which form tough, leathery mats. Cyanobacterial photosynthesis presents the main input of organic material which finally stipulates the presence of other groups of microorganisms. The dead organic matter is mineralized by chemoorganotrophic bacteria. First, this is an aerobic process but with increasing oxygen depletion the environment becomes anaerobic. The final mineralization step is carried out mainly by sulfate-reducing bacteria. These bacteria oxidize simple organic compounds by reducing sulfate to sulfide. The sulfide is eventually oxidized by anoxyphotobacteria, namely the purple sulfur bacteria. The layer of the sulfate-reducing bacteria is characterized by a black color, due to precipitated amorphous FeS. Vertically stratified populations of cyanobacteria, purple sulfur bacteria and sulfate-reducing bacteria characterize well-developed microbial mats on the Mellum island. The steep physico-chemical gradients in these mats are the resultant of the growth and metabolic activities of the contributing organ-

isms.

Although, at first sight, the cyanobacterial community seemed rather diverse, it was found that only two species were of particular interest. The filamentous *Oscillatoria* sp. was the organism that initially colonized the sediment. In a later stage of mat development *Microcoleus chthonoplastes* became a dominant organism. The latter was the real mat builder. *Oscillatoria* sp., however, usually is present and sometimes may even become dominant in the *M. chthonoplastes*-mat.

The measurements of nitrogenase activity in the microbial mat showed a close association with the layer of cyanobacteria. This was astonishing inasmuch no heterocyst-forming could be detected by microscopic examination of samples of the mat. Nitrogenase, the enzyme responsible for nitrogen fixation, is extremely sensitive to oxygen and therefore aerobic organisms have to possess oxygen protection mechanisms. Certain species of the aerobic and oxygenic phototrophic cyanobacteria form specialized cells, the heterocyst. The heterocyst does not evolve oxygen by photosynthesis and this cell is the site of nitrogen fixation. The measurements of nitrogenase activity in the microbial mat showed a close association with the layer of cyanobacteria. This was astonishing inasmuch no heterocyst-forming cyanobacteria could be detected

Young mat systems, dominated by *Oscillatoria* sp. showed a specific nitrogenase activity (expressed as mg chlorophyll) which was considerably higher than in established mats. By isolating *Oscillatoria* sp. in pure culture, it could be shown that this organism grew well in the absence of combined nitrogen and that it possessed high nitrogenase activity. The mechanisms by which the cyanobacteria overcome the problems related to nitrogenase protection against oxygen inactivation were studied in the field and in the laboratory.

In a vertical gradient in the sediment, it was shown that maximum nitrogenase activity occurred in a depth of 2 - 3 mm. Oxygen-profiles, measured by using micro-electrodes, have shown that in the light the concentration of oxygen was maximal in the upper 1-mm of the sediment, whereas below 1.5 mm depth virtually no oxygen could be detected. Moreover, only light of long wavelength penetrated the cyanobacterial mat relatively well. The light that reaches the lower layers of the cyanobacterial mat, certainly does not support oxygenic photosynthesis but presumably allows non-cyclic electron transport through photosystem I. Measurements of oxygen concentration have also shown that during the dark period the sediment turns anaerobic up to the mat surface. Acetylene reduction measured repetitively during a period of 30 h showed a high activity at sunrise. The sediment probably is still anaerobic in the early morning, whereas the low light intensity presumably does not support very active oxygenic photosynthesis. The presence of low light intensity and anaerobic conditions in the sediment appeared to stimulate nitrogen fixation.

In summary, these observations were taken as evidence that the non-heterocystous cyanobacterium *Oscillatoria* sp. was responsible for the bulk of the observed nitrogenase activity. Many other cyanobacteria were isolated from the same environment. It was striking that also several heterocystous cyano-

bacteria were isolated after enrichment on media deprived of combined nitrogen. The reasons why these organisms are of no importance in the Mellum mats, are still obscure. Apparently, the heterocystous cyanobacteria are outcompeted due to extremely fluctuating environmental conditions. Several other unicellular and filamentous cyanobacteria were isolated and some of these organisms possessed the capacity to synthesize nitrogenase anaerobically. *M. chthonoplastes* also fixed nitrogen anaerobically. However, taking into account that induction of nitrogenase showed a lag of at least 8 h, it was doubted that this organism will considerably contribute to overall nitrogen fixation.

Growing *Oscillatoria* sp. in the laboratory under light-dark cycles, nitrogen fixation predominantly occurred in the dark. The light-dark cycle offered the organism the possibility to separate nitrogen fixation temporally from oxygenic photosynthesis. However, it was observed that, using short dark periods, nitrogenase appeared already in the light before the onset of the dark period. Transferring a culture, grown under a light-dark cycle, to continuous light, nitrogen fixation continued also in the light. Moreover, cultures grown under continuous light also showed a discontinuous nitrogenase activity. Therefore, a light-dark cycle is not an absolute prerequisite. Nitrogen fixation and oxygenic photosynthesis seem to be incompatible processes. In the absence of combined nitrogen the cyanobacterium degrades the phycobiliproteins, the accessory pigments of photosystem II. When a certain degree of phycobiliprotein degradation is reached, the activity of the oxygenic photosystem II is lost. As soon as the evolution of oxygen has stopped, nitrogenase is synthesized and the phycobiliproteins are resynthesized. Then, if sufficient nitrogen is fixed, oxygenic photosynthesis resumes and nitrogenase activity disappears.

The temporal separation of oxygenic photosynthesis and nitrogen fixation is an important mechanism in non-heterocystous cyanobacteria. However, this mechanism alone cannot explain nitrogen fixation in these cyanobacteria because nitrogenase must also be protected against atmospheric oxygen. Several mechanisms may be considered. There is no doubt that a continuous loss of nitrogenase activity occurs under aerobic conditions. However, a high rate of synthesis of the enzyme apparently counteracts losses of activity, resulting in net nitrogen fixation. Additionally, a switch-off mechanism on the molecular level might protect the enzyme during oxygen stress. During the dark period, and also in the light when photosynthetic activity stops, respiration probably may contribute to keep the intracellular oxygen concentration low.

Usually, cyanobacteria survive dark periods by respiration of their endogenous carbon reserves (glycogen). Microbial mats on Mellum island become anaerobic during the night. Therefore, the cyanobacteria that occur in these mats must possess mechanisms to generate energy anaerobically in the dark, at least for the purpose of maintenance. Furthermore, and in contrast with other cyanobacteria, *Oscillatoria* sp. was shown to be able to fix nitrogen under dark anaerobic conditions. This also indicated that anaerobic dark energy metabolism in this organism is possible. *Oscillatoria* sp. fermented endogenous carbon reserves into lactate and ethanol in a heterofermentative pathway. In

the presence of elemental sulfur, lactate fermentation was repressed and sulfur was reduced to sulfide. However, when the cells contained nitrogenase, sulfur reduction did not occur. Unless a nitrogenase-reducible substrate was present, lactate fermentation under such conditions also occurred in the presence of elemental sulfur. Cells containing nitrogenase reduced acetylene to ethylene and then no lactate fermentation was observed. The repression of sulfur reduction by nitrogenase is presently not understood.

M. chthonoplastes only degraded glycogen anaerobically in the dark in the presence of elemental sulfur, producing sulfide. Except for *Oscillatoria* sp. none of the Mellum strains tested possessed fermentative pathways. Several - but not all - strains were able to reduce elemental sulfur.

The capacity to fix nitrogen under aerobic conditions and its astonishing metabolic flexibility allowed *Oscillatoria* sp. to colonize an extremely low nutrient environment.